

REVIEW

Live Attenuated Influenza Vaccine - A review

R. GASPARINI, D. AMICIZIA, P.L. LAI, D. PANATTO
Department of Health Sciences, University of Genoa, Italy

Key words

Influenza • Vaccine • Live Attenuated Influenza Vaccine (LAIV) • Inactivated Influenza Vaccine (IIV)

Summary

Owing to the variability of influenza viruses, vaccine composition needs to be up-dated annually. As many variables can influence their efficacy, vaccines are still considered "sub-optimal". Many studies have been carried out in recent years to improve vaccines. In particular, researchers and vaccine-producing corporations have focused on developing a live vaccine.

Among the candidate vaccines, the strain developed by Maassab has recently been licensed in the USA and Europe, after extensive investigation. This vaccine is safe and well tolerated, and has shown very good genetic stability. Although vaccine recipi-

ents are able to spread the virus, transmission to close contacts is practically non-existent.

Studies on cold-adapted attenuated influenza vaccines have demonstrated that such vaccines are effective, and sometimes more effective than inactivated influenza vaccines. Cold-adapted attenuated influenza vaccines therefore appear to be an important weapon against influenza. However, a more widespread use of these vaccines is to be recommended, especially in children, as the more acceptable way of administration can favour parental compliance.

Introduction

Owing to the variability of influenza viruses, vaccines need to be up-dated annually. Particularly, subjects at risk must be vaccinated every year. However, many variables may influence vaccine efficacy, such as matching with circulating viruses, age, concomitant pathologies, etc. Influenza vaccines are therefore considered to have a suboptimal efficacy profile. For these reasons, many improvements, such as the addition of adjuvants, have been made in recent years. Researchers have long been trying to develop a safe and effective live attenuated influenza vaccine. Indeed, considerable attention has recently been devoted to the challenge of generating effective immune responses at mucosal surfaces. Mucosal surfaces are sites of pathogen entry and replication, making immune responses at these locations critical for effective immunity [1].

Candidate influenza vaccines delivered intranasally and inducing mucosal immunity include live attenuated viruses [2], adjuvanted killed vaccines [3, 4], and recombinant viral vectors [5]. Live attenuated vaccines are prepared through the reassortment of a donor (master) strain with lesions in internal antigen genes and an epidemic strain. Three approaches have been attempted with the aim of creating a suitable vaccine, namely: developing temperature-sensitive (ts) mutants by treatment with 5-fluorouracil [6], selecting host-range variants by adaptation in non-human cells [7], and adapting the influenza virus to grow at low temperature [8-10].

Immunoresponse against Influenza viruses

In most developed countries, inactivated egg-derived vaccines are currently used. However, these have some drawbacks, such as low efficacy in the elderly and in the event of pandemics. Furthermore, the acceptance of an inactivated influenza vaccine (IIV) may be conditioned by their intramuscular administration, particularly in children. The aim of vaccination with a live attenuated virus is to induce a secretory and systemic immune response that more closely resembles the immune response detected after natural infection.

Resistance to influenza virus infection is due to both mucosal and systemic immunity [11-14]. Moreover, antibodies generated locally in the upper airways constitute the main defence against natural contagion [12-14]. While secretory immunoglobulin A (SIgA) is involved in the defence of the upper respiratory tract, serum immunoglobulin G (IgG) protects the lower airways. Spontaneous infection may confer long-term immunity against influenza viruses [15]. In addition, the cell-mediated immune response is principally responsible for eliminating virus-infected cells, thus facilitating recovery from the disease. Human infection generates antibodies against all the most important viral antigens [16-17]. Whereas antibodies against the inner components, matrix protein (M) and nucleoprotein (NP), are not protective [18], antibodies against hemagglutinin (HA) and neuroaminidase (NA) are associated with resistance to infection. As most microorganisms enter the human body through mucosal tissue, the local immune system constitutes the first line of defence against infection, together with innate immunity. On first infection, immunoglobulin M

(IgM), IgG and immunoglobulin A (IgA) against HA are present in nasal secretions [11-19]. While IgGs are derived as a serum transudate, IgA and IgM are generated locally. Resistance to influenza viruses or disease is linked with the amount of local and/or serum antibody against HA and NA [18].

The killing of influenza-infected cells is caused by cytotoxic T cells (CTLs), jointly with influenza-specific antibodies and complement [20-23]. Influenza infection elicits a robust T-helper response, which has a valuable function in rousing antibody production against the virus [24, 25].

Historical remarks

More than 40 years have passed since the first cold-adapted influenza viruses were developed in Russia as potential live attenuated vaccines.

Therefore, the strategy of creating live influenza vaccines has been developed around a two-step process. The first phase requires the creation of so-called 'master strains' of attenuated vaccines. Once acceptable 'master strains' are available, this stage rarely needs to be repeated. The second phase involves the genetic transfer of the attenuation characteristics from the older 'master strains' to newer variants that emerge from the latest outbreaks. This phase would need to be constantly repeated in a reproducible manner, in order to update the vaccine [26]. Development of the Russian live attenuated 'master strains' began with much the same traditional approach as for most other already used live vaccines. This involved repeated passages of an initial virus believed to represent the 'wild-type' in the laboratory host system, with a view to selecting a less virulent strain.

Smorodincev [27] and Alexandrova et al. [8] were the key Russian scientists who developed the cold-adapted 'master strains' [26]. Wild-type virus isolates were passaged in embryonate eggs at successively lower temperatures than that normally used for their replication in the laboratory. Finally, viruses were produced which grew well at a temperature of 25°C, whereas the parent virus hardly grows at all at this temperature.

Researchers in the USA have demonstrated the feasibility of live cold-adapted vaccine preparation. The vast majority of clones recovered from mixed infections (donor and wild-strain) in which selective measures were used, as described above, were found to have only the HA and NA genes from the wild-type virus, but the other 6 genes from the cold-adapted parent ('6-2' gene composition). This work was originally carried out by comparing the mobility of individual nucleic acid segments during gel electrophoresis; now, however, it can be done by applying polymerase chain reaction methods. Such '6-2' reassortant viruses retain the properties of growing well at 25°C and poorly at 39°C, as is the case with their attenuated parent; however, they contain the surface antigens of new epidemic strains.

Among cold-adapted strains, the best strains were developed by Maassab [9]. Before 1967, the attenuation

of influenza viruses was achieved through a gradual process of cold adaptation. Subsequently, Maassab et al. optimized attenuation by abruptly reducing the influenza virus cultivation temperature from 35 to 25°C. In this way, adaptation took place in weeks instead of 6-8 months [28]. An attenuated Hong Kong strain of influenza virus (A2/Aichi/2/68) was successfully used in clinical trials by Maassab et al. [28]. After this research, many other studies were carried out using the cold-adapted influenza virus [29, 30], until a cooperative program among vaccine evaluation units that was sponsored by the National Institute of Allergy and Infectious Diseases (NIAID) brought these vaccines to complete development by Wyeth-Ayerst Laboratories (Philadelphia, PA) [26].

Safety and tolerability

Maassab's study, which was published in 1969, reported that the A2/Aichi/2/68 cold-adapted attenuated influenza virus was genetically stable, safe and well tolerated in mice, ferrets and humans [29]. Subsequently, Murphy et al. repeatedly demonstrated [29, 31, 32] that different cold-adapted influenza viruses attenuated with the methods of Maassab and Cox induced only febrile systemic illness.

Likewise, Alexandrova et al. demonstrated that two recombinant cold-adapted attenuated Influenza A vaccines were genetically stable, safe and well tolerated in children aged 3-15 years [33].

In 1992, Anderson et al. [30] published the results of a clinical trial conducted on children aged 8 months to 14 years with a cold-adapted influenza B/Texas/84 reassortant strain. The virus proved phenotypically stable and the study demonstrated the safety of the vaccine. Live attenuated, cold-adapted monovalent and bivalent influenza A vaccines were evaluated in seronegative infants (6-18 months) by Gruber et al. [34]. The Authors observed no excess of fever or respiratory symptoms in vaccine recipients than in controls. The following year, the same Authors published the results of an analogous study [35] conducted on a larger number of subjects (1,126 children) with a wider age range (2 months, 3 years). The vaccine was well tolerated, and fever and respiratory symptoms, including rhinorrhea, were not statistically more frequent in recipients than in controls. In 1997, King et al. reported the results of a trial conducted in children aged 18-71 months [36] with various doses of a trivalent cold-adapted influenza vaccine (CAIV-T) administered intranasally by means of drops or spray. The results confirmed that, at all doses, the vaccine was safe and well tolerated.

In 1996-1997, Piedra et al. performed a very interesting study on the safety and tolerability of a CAIV-T in children aged 15-71 months [37]. They subsequently studied the safety of sequential annual doses of CAIV-T over a 4-year period in the same cohort of volunteers. During the first year of study, 1,602 children were recruited (1,314 received 2 doses and 288 one dose of

the attenuated vaccine or placebo in a 2:1 ratio). In the second year, 1,358 of the original participants received 1 dose of vaccine or placebo, according to the original treatment. In the third and fourth years, subjects who had previously been given the cold-adapted vaccine were requested to receive their 3rd or 4th dose of the vaccine: 642 and 549 subjects were enrolled, respectively. The Authors concluded that CAIV-T was safe in children. However, mild respiratory symptoms (particularly runny nose), and gastrointestinal and systemic symptoms of short duration were observed in a minority of recipients, particularly after the first dose of the vaccine.

In the early 2000s, convincing evidence of the safety and efficacy of cold-adapted live influenza vaccines was provided by many published papers, notably that of Beyer et al., who carried out a meta-analysis of 19 studies conducted in the United Kingdom (UK) and USA [38]. With regard to safety, these Authors considered 11 clinical trials involving 4,088 subjects, and found that the frequency of systemic vaccine reactions ranged from 0 to 31.6%. In the trials considered, the vaccine recipients received an attenuated cold-adapted vaccine or an inactivated influenza vaccine. None of the 11 comparisons showed any significant difference between the two vaccines.

In 2003, Harper et al. prepared a summary of the recommendations of the Advisory Committee on Immunization Practices (ACIP) of the US Center for Diseases Control and Prevention (CDC) on the use of the attenuated Influenza vaccine (LAIV) [39]. These Authors underlined that the LAIV was approved in the US for healthy persons aged 5-49 years only, and subdivided the safety data on the basis of the age of the population. The symptoms more often reported in vaccinated children than in placebo recipients were: runny nose, headache, fever, vomiting, abdominal pain and myalgia. These symptoms were associated more frequently with the first dose and were self-limiting [40, 41]. Nevertheless, the Authors pointed out that unpublished data from a study including subjects from 1 to 17 years indicated an increase of symptoms in the subset of subjects aged 12-59 months, and that the vaccine should be licensed only for children over 60 months of age. Regarding adults, Harper et al. emphasized that nasal congestion, headache and sore throat had been reported more often in vaccine recipients than in placebo recipients [39].

Bergen et al. [42] performed a specific safety study on attenuated influenza vaccine on a large sample of children and adolescents (12 months-17 years). A total of 9,689 evaluable subjects were recruited in the clinical trial. The conclusions of the Authors were that Cold-Adapted Influenza Vaccine (CAIV) was generally safe in children and adolescents; however, there was an increased risk of asthma/reactive airway disease in children < 36 months of age.

In 2004, a paper [43] reported that CAIV-T had been evaluated in 20 clinical trials, in which 20,000 subjects (more than 15,000 children and adolescents aged 1-17 years and more than 3,700 healthy adults aged 18-64 years) had received more than 28,000 doses of vaccine,

and had been compared with more than 11,300 placebo recipients. The Author reported that CAIV had been well tolerated in clinical trials with 19 different 6:2 reassortant virus strains, both of type A (H1N1 and H3N2) and of type B, and underlined that no severe adverse events (SAEs) were associated with vaccination, even in infants and children. However, Belshe also reported that mild, transient symptoms, such as mild upper respiratory signs, low fever and decreased activity, had been described more frequently in vaccinated subjects than in placebo recipients in several clinical trials. In 2006, Belshe's investigation was up-dated by Ambrose et al. [44]. These Authors evaluated the results obtained in more than 40,000 vaccinated subjects, and confirmed the data reported by Belshe.

In 2007, Belshe et al. published a study carried out on children aged 6 to 59 months, without a recent episode of wheezing illness or severe asthma, who were randomly assigned in a 1:1 ratio to receive either cold-adapted Trivalent live attenuated Influenza Vaccine or TIV [45]. Influenza-like illness was monitored by means of cells cultures throughout the 2004-2005 influenza season. Safety data were available for 8,352 children. Among previously unvaccinated children, wheezing within 42 days after the administration of dose 1 was more common with live attenuated vaccine than with inactivated vaccine, primarily among children aged 6 to 11 months. Rates of hospitalization for any cause during the 180 days after vaccination were higher among the live attenuated vaccine recipients aged 6 to 11 months than among the recipients of inactivated vaccine in this age-group (6.1% vs. 2.6%, $p = 0.002$).

Recently, Tennis et al. have published the results of a post-marketing evaluation of the frequency and safety of live attenuated influenza vaccine use in non-recommended children younger than 5 years [46]. The 2007 US approval for the use of LAIV in children aged 24-59 months included precautions against use in: children < 24 months (cohort 1), children aged 24-59 months with asthma (cohort 2), and those with recurrent wheezing (cohort 3) or altered immunocompetence (cohort 4). A post-marketing commitment was initiated to monitor LAIV use and the frequency of selected safety outcomes in these cohorts. Vaccination rates and the frequency of hospitalizations or emergency department visits within 42 days after LAIV and TIV administration were estimated from health insurance claims from 2007 to 2009. Rates of LAIV use per 10,000 child-days were lower among cohorts 1, 2, and 4 than among the LAIV-recommended population.

In cohort 3, however, rates of LAIV use per 10,000 child-days were similar to those of the LAIV-recommended population. The rate of emergency department visits/hospitalizations within 42 days of vaccination with LAIV was the same as or less than the rate within 42 days of vaccination with TIV.

A very recent global evaluation of data on the safety and tolerability of LAIV was published by Ambrose et al. in 2008 (49,000 individuals in 48 completed studies, including more than 18,000 children younger than

5 years) [47]. At that time more than 10 million doses had been commercially distributed in the United States since licensure. LAIV are intended not only to mitigate seasonal influenza, but also to guard against pandemics. In this regard, Mallory et al. [48] evaluated the safety, tolerability and immunogenicity of a monovalent intranasal 2009 A/H1N1v (A/California/07/09) LAIV in children and adults. Solicited symptoms were less frequent after dose 2 in both adults and children, and no vaccine-related serious adverse events occurred.

As already mentioned [42], a particular concern of LAIV vaccination is the possibility that it may give rise to wheezing and asthma attacks in children aged < 36 months. For this reason, the CDC contraindicate the vaccination of FluMist® in children under 2 years and in those younger than 5 years with asthma or who have had one or more episodes of wheezing within the past year [49]. For the same reason, in 2011 the EMA recommended that marketing authorization be granted for LAIV (Fluenz®) in healthy individuals aged from 24 months to 18 years [50]. Until 2006, only a few studies had been carried out on children with asthma [51]. In 2006, however, Fleming et al. [52] published the results of a large trial in which the efficacy and safety of LAIV were compared with those of TIV in children (aged > 6 years) and adolescents (aged < 17 years) with asthma. The study involved 1,111 subjects, who received the LAIV, and 1,109 subjects who received the TIV. The Authors concluded that the CAIV-T was well tolerated in children and adolescent with asthma, and that adverse pulmonary outcomes were not significantly more frequent among CAIV-T recipients than TIV recipients.

PHENOTYPIC AND GENOTYPIC STABILITY OF LAIV, AND VIRAL SHEDDING

Closely related with the safety of LAIV is the phenotypic and genetic stability of the attenuated strain.

To characterize genetic markers of attenuation, Maassab et al. drew up a set of genetic markers: growth of the cold-adapted strain at 25, 35 and 41°C, replication of the strain over different ranges of pH of the medium (5.7-6.3 and 7.0-7.2), and characteristics of plaque formation in culture cells at 35 and 25°C. They also verified the avirulence of the attenuated strain in ferrets and mice [9]. Similar techniques, particularly the plaque formation test, were also used to verify the attenuation of the Russian cold-adapted virus genetically [53].

The most important issue regarding the attenuated genes was their genetic stability; indeed, it was feared that the vaccine virus could revert to the wild type. In 1999, Cha et al. [54] carried out a genotyping study on 18 attenuated strains isolated from secretions of 17 participants in a clinical trials, who had received the cold-adapted attenuated vaccine. Analysis of 11,800 nucleotides demonstrated that, even after replication in human hosts, the cold-adapted strains remained stable [10, 54].

Another important issue regarding the attenuated strains was viral shedding, and thus the possibility of transmission between vaccine recipients and close contacts. Viral shedding after the administration of cold-adapted

attenuated vaccine has often been demonstrated, both in adults and in children. While the quantity of virus shed by adults is usually lower than the minimal infective dose [55], the issue is not completely clear with regard to children [2, 56]. However, a large study carried out on vaccinated children showed that transmission practically did not occur [10, 57].

IMMUNOGENICITY, EFFICACY AND EFFECTIVENESS

Smorodincev published a short review on the efficacy of Russian Live Influenza Vaccines (LIV) in 1969 [27]. The Author pointed out that, when the vaccine matched with circulating strains and when vaccinated subjects were properly studied, morbidity was clearly lower among the recipients of the cold-adapted vaccine than in a control group of volunteers.

In 1969, Maassab et al. administered cold-adapted attenuated influenza vaccine to 10 volunteers [9]. Seroconversion was observed in two recipients who were seronegative before administration, and a 4-fold or more increase in HI titers was observed in subjects previously immunized (previously documented illness or vaccination) with the same strain contained in the vaccine.

A trial involving wild-type or cold-adapted influenza A/Alaska/6/77 virus strains was conducted by Murphy et al. [29]. In all of the groups studied, administration of the attenuated or the wild-type parent strain elicited an immunoresponse. However, the attenuated virus elicited a lesser immunoresponse than the wild-type strain.

The immunogenicity and protection provided by a recombinant obtained from an H3N2 strain (A/Victoria/75) and a cold-adapted donor A/Ann Arbor/6/60 (H2N2) were studied in healthy young adult males [58]. A serum-neutralizing immunoresponse was elicited in 90% of vaccinees. Furthermore, the recipients of the attenuated vaccine displayed significant protection in comparison with unvaccinated.

In the 1996-1997 influenza season, Belshe et al. [40] conducted a clinical trial using a cold-adapted, trivalent influenza virus vaccine. One dose of vaccine or placebo was administered by intranasal spray to 288 children, while 1,314 children received two doses 60 days apart. Children initially seronegative showed a 4-fold increase in antibody titers in 61-96% of cases, depending on the influenza strain. The volunteers were followed up for two influenza seasons. Influenza confirmed by cell-culture isolation was significantly more frequent in the placebo group than in the vaccine group (vaccine efficacy 93% [CI-95%: 88-96%]). The vaccine was effective against 2 influenza wild strains (A-H3N2 and B) circulating in the 1996-97 winter. The one-dose regimen displayed 89% efficacy and the two-dose regimen 94% efficacy.

Just before the authorization of Flumist®, Beyer et al. carried out a meta-analysis of 19 studies comparing a cold-adapted attenuated live influenza vaccine with inactivated vaccine [38].

Regarding the immunoresponse against H3N2 viruses, inactivated influenza vaccines (IIV) elicited twice as many antibodies as LIV; the immunoresponse to H1N1 strains

was similar, though slightly better for IIV. Only a few studies assessed secretory IgA. Although the literature on this issue was scant, the papers considered by Beyer et al. demonstrated a stronger IgA production by LIV recipients than IIV recipients. The papers examined in the meta-analysis also included efficacy assessment through the virological follow-up of the volunteers, which was performed by isolating influenza virus in cell-cultures during the winter season. This evaluation showed that the protection conferred by the two vaccines was similar, though LIV yielded slightly better results than IIV. Regarding this last issue, Belshe et al. carried out a trial in children in 2004-05 [45]. They found fewer culture-confirmed cases of influenza in the recipients of the live attenuated vaccine than in those who received inactivated vaccine (153 vs. 338 cases, $p < 0.001$). The superiority of the attenuated vaccine was confirmed on considering both antigenically well-matched and drifted viruses. Similarly, in children (6-71 months) with recurrent respiratory tract infections, Ashkenazi et al. compared recipients of CAIV-T ($n = 1,050$) with recipients of TIV (1,035), and found that the attenuated vaccine displayed greater efficacy than the inactivated vaccine [59].

On vaccinating children and adolescents with asthma (6-17 years of age), Fleming et al. also observed that CAIV-T conferred greater protection against community-acquired culture-confirmed influenza than TIV [52]. Finally, an H1N1v pandemic live attenuated vaccine was evaluated by Mallory et al. [48] in children (H1N1v LAIV, $n = 261$; placebo, $n = 65$) and adults (H1N1v LAIV, $n = 240$; placebo, $n = 60$). After 2 administrations (28 days apart), LAIV displayed an immunogenicity profile similar to that of the previous seasonal live cold-adapted influenza vaccine. Indeed, after 2 doses, sero-response rates were 32% (vaccinees) vs. 14.5% (placebo recipients), and 14.9% (vaccinees) vs. 5.6% (placebo recipients) in adults and children, respectively.

Discussion

Live attenuated vaccines can significantly contribute to the control of seasonal and pandemic influenza episodes. However, the vaccine must have some important characteristics. Obviously, it must be safe and effective. The genetic stability of attenuated strains and their possible transmission to contacts are closely related to safety. Several studies have demonstrated that vaccine viruses do not revert to wild-type virus [54, 55].

Other concerns regard the use of attenuated viruses in the elderly, in whom previous frequent contact with influenza viruses could determine an immune pressure and facilitate reversion to the wild type. Similarly, the use of a live vaccine to prevent a pandemic could generate new variants. However, if a reassortment of an attenuated virus and a wild pandemic strain occurred, the resulting reassortant would probably be less pathogenic in humans. Another worry about live vaccines is safety. Since licensure in the USA in 2003, General Practitioners (GPs) and nurses have considered safety concerns to be the

foremost reason for the low acceptance of the vaccine. However, many of the doubts seem to stem from unproven fears and misperceptions rather than from recognized figures [10].

Since LAIV has proved to be safe and effective, a widespread use of this vaccine achieve better control of influenza among children, who are the first target of novel strains. Furthermore, children are important spreaders of wild viruses. Full acceptance of the live attenuated vaccine could provide further protection through herd immunity, as both the "Tecumseh Study" [60] and the Japanese experience [61] have demonstrated.

In addition, recent progress in genetic engineering could ameliorate the preparation of live influenza attenuated vaccines. Indeed, Jung et al. recently published a study on a useful method of preparing live attenuated influenza virus [62]. Using reverse genetics, these Authors were able to generate "6 + 2" influenza vaccine strains corresponding to A/Chicken/Korea/MS96 (H9N2) and A/Indonesia/5/2005 (H5N1). Furthermore, Solorzano et al. used reverse genetics to generate a live-attenuated vaccine against the H1N1v pandemic strain. These Authors placed particular emphasis on the polymerase genes [63]. Reverse genetics is less laborious and less time-consuming than the traditional method of attenuation.

Once the high pathogenicity genes of the influenza strains are better known, reverse genetics will further improve the preparation of influenza vaccines. As yet, the molecular basis of virulence among influenza viruses is only partially recognized; for instance, it is known that the high pathogenicity of H5N1 strains is determined by basic amino acids at the cleavage site of hemagglutinin, and also by the presence of lysine (instead of glutamic acid) in position 627 of the PB2 protein coded by the corresponding gene [64].

In conclusion it can be asserted that:

- the safety profile of LAIV is well consolidated, and precautions must be taken only in infants (< 24 months of age) and children under 5 years with a history of asthma;
- LAIV has been safely administered both to HIV-infected children and adults [65, 66];
- the donor strains have excellent genetic stability;
- transmission to close contacts is practically non-existent;
- LAIV is well tolerated;
- the attenuated cold-adapted vaccine has been more effective than inactivated influenza vaccines in some circumstances in children [59];
- as LAIV also stimulates cellular immunity, it could help to reduce complications in infants and children;
- LAIV is also useful in preventing otitis media in children [67];
- the nasal spray is clearly more acceptable than parenteral administration;
- LAIV stimulates local immunity, and probably provides broad cross-protection;
- the administration of LAIV is very flexible; Breiman et al. [68] recently demonstrated that LAIV could be co-administered with oral polio vaccines.

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■ Correspondence: Roberto Gasparini, Department of Health Sciences, University of Genoa, via Pastore 1, 16132 Genoa, Italy - E-mail: gasparini@unige.it